

Pathogen profile

Potato spindle tuber viroid: the simplicity paradox resolved?

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SUMMARY

Taxonomy: Potato spindle tuber viroid (PSTVd) is the type species of the genus *Posipiviroid*, family *Pospiviroidae*. An absence of hammerhead ribozymes and the presence of a 'central conserved region' distinguish PSTVd and related viroids from members of a second viroid family, the *Avsunviroidae*.

Physical properties: Viroids are small, unencapsidated, circular, single-stranded RNA molecules which replicate autonomously when inoculated into host plants. Because viroids are non-protein-coding RNAs, designation of the more abundant, highly infectious polarity strand as the positive strand is arbitrary. PSTVd assumes a rod-like, highly structured conformation that is resistant to nuclease degradation *in vitro*. Naturally occurring sequence variants of PSTVd range in size from 356 to 361 nt.

Hosts and symptoms: The natural host range of PSTVd—cultivated potato, certain other *Solanum* spp., and avocado—appears to be quite limited. Foliar symptoms in potato are often obscure, and the severity of tuber symptoms (elongation with the appearance of prominent bud scales/eyebrows and growth cracks) depends on both temperature and length of infection. PSTVd has a broad experimental host range, especially among solanaceous species, and strains are classified as mild, intermediate or severe based upon the symptoms observed in sensitive tomato cultivars. These symptoms include shortening of internodes, petioles and mid-ribs, severe epinasty and wrinkling of the leaves, and necrosis of mid-ribs, petioles and stems.

INTRODUCTION

The 1971 discovery of potato spindle tuber viroid (PSTVd) by T. O. Diener was a landmark event in modern biology. Viroids are the smallest known agents of infectious disease—small (246–401 nt), highly structured, circular, single-stranded RNAs that lack detectable messenger RNA activity. Whereas viruses supply some or most of the genetic information required for their

replication, viroids can be regarded as 'obligate parasites of the cell's transcriptional machinery'.

Prior to the discovery of PSTVd, many biologists believed that autonomous replication of a pathogen required a minimum genome size of at least 1×10^6 Da, a value derived from the genome size of the smallest RNA bacteriophage. The significance of viroids for molecular biology was first summarized by Diener nearly 20 years ago as a series of questions:

- 1 What are the molecular signals that induce certain host DNA-dependent RNA polymerases to accept viroids as templates for the synthesis of complementary RNAs?
- 2 Are the molecular mechanisms responsible for viroid replication operative in uninfected cells? If so, what are their functions?
- 3 How do viroids induce disease? In the absence of viroid-specified proteins, disease must arise from the direct interaction of host cell constituents with either viroids themselves or viroid-derived RNAs.
- 4 What determines viroid host range? In the broadest terms, are viroids restricted to higher plants? Do they have counterparts in animals?

Over the succeeding years, much has been learned about the molecular biology of PSTVd and its interaction with host plants; nevertheless, the precise nature of the molecular signals that allow PSTVd to replicate autonomously and induce disease remains elusive. Additional information about PSTVd biology and molecular biology can be found in a recent monograph on viroids (Hadidi *et al.*, 2003) as well as several more specialized reviews (e.g. Daros *et al.*, 2006; Ding *et al.*, 2005; Flores *et al.*, 2005; Tabler and Tsagris, 2004; Ding and Itaya, 2007).

POTATO SPINDLE TUBER DISEASE

Potato spindle tuber disease was first described by Martin (1922) who reported a 'new potato trouble' affecting seed potato production in New Jersey; shortly thereafter, its infectious nature and ability to spread in the field led Schultz and Folsom (1923) to group spindle tuber with several other 'degeneration diseases' of potatoes. Figure 1 shows the symptoms of potato spindle tuber disease in the variety Kennebec.

Nearly 50 years were to elapse before Diener's (1971) demonstration that the molecular properties of the causal agent were

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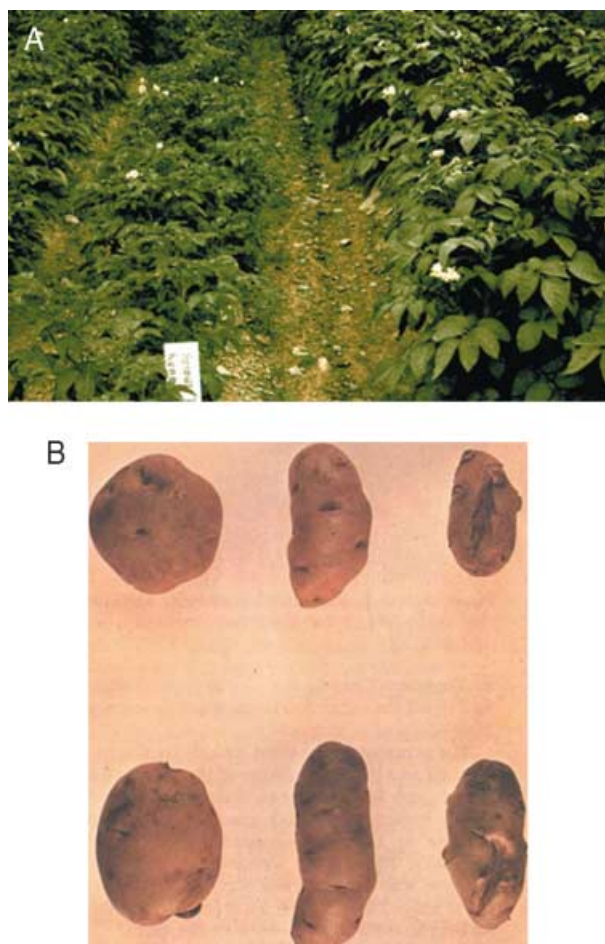


Fig. 1 Symptoms of potato spindle tuber disease under field conditions. (A) Foliar symptoms in the potato variety Kennebec. Note the stunted, unthrifty appearance of the PSTVd-infected plants (left) compared with the uninfect controls (right). (B) Tuber symptoms. Left to right, healthy tubers, tubers infected with a mild-intermediate strain of PSTVd, tubers infected with 'unmottled curly dwarf' (presumably a severe strain of PSTVd). [Reprinted from U.S. Department of Agriculture, Agricultural Research Service Agriculture Handbook no. 474 (Potato Diseases) by M. J. O'Brien & A. E. Rich, 1976].

fundamentally different than those of conventional plant viruses like potato virus X or potato virus Y. Systematic studies of the properties of PSTVd became possible only after the 1962 report by Raymer and O'Brien that the spindle tuber agent is able to infect tomato (*Lycopersicon esculentum* Mill) where it produces a characteristic disease syndrome (e.g. stunting and epinasty) not easily confused with those of other known potato or tomato pathogens. A review by Diener (2003) provides a personal perspective on the discovery of PSTVd.

At one time, spindle tuber disease was common in potato-growing regions of the northern United States, Canada and western Europe. Indexing on Rutgers tomato greatly facilitated PSTVd

diagnosis, but the existence of many strains that produce only mild or indistinct symptoms in tomato complicated efforts to eliminate infected material from seed production programmes and germplasm collections. The PAGE method for PSTVd detection developed by Morris and Wright (1975) represented a considerable improvement on the tomato bioassay, because, in addition to distinguishing viroid infected from health samples, such a test can also be used to differentiate between different viroids and viroid strains (Singh and Boucher, 1987). It was, however, the use of recombinant DNA technology to develop a simple and reliable 'dot blot' hybridization test for PSTVd (Owens and Diener, 1981) that made large-scale screening programmes practical. More recently, a variety of PCR-based methods for PSTVd detection including real-time RT-PCR have been developed (e.g. Boonham *et al.*, 2004). As a result, PSTVd has been virtually eliminated from potato breeding programmes and commercial production in North America and Europe, thereby illustrating the value of combining rapid and sensitive diagnostic methods with a rigorous clean stock or seed certification scheme.

PSTVd is readily transmitted mechanically and, in the case of potato or tomato, is also transmitted through both botanical seed and pollen. Although not usually considered insect-transmissible, the aphid vector *Myzus persicae* can efficiently transmit PSTVd if potato plants are coinfectd with potato leafroll virus (PLRV). Querci *et al.* (1997) have shown that PSTVd is heterologously encapsidated within the particles of PLRV, a phenomenon that may have important implications for the epidemiology and spread of PSTVd under field conditions.

Over the past 20 years, repeated disease outbreaks involving PSTVd or other, closely related pospiviroids have been reported in tomatoes growing in commercial greenhouses. Several of the PSTVd isolates involved share a common origin, possibly in Oceania (Verhoeven *et al.*, 2004) and other solanaceous species harbouring latent infections by PSTVd may have provided the initial inoculum for these outbreaks (Behjatnia *et al.*, 1996; Puchta *et al.*, 1990). Molecular detection methods have clearly improved our ability to detect and eliminate viroid-infected plants, but questions that need to be addressed if new outbreaks are to prevented include: Have there been certain changes in agricultural/greenhouse practices (e.g. growing crops that may be symptomless viroid carriers next to susceptible crops) that can explain the appearance of new infections? Should these practices be modified to avoid chance infections of cultivated crops?

The origin of PSTVd and the other pospiviroids that infect solanaceous crop species is not known, but Martinez-Soriano *et al.* (1996) have proposed that commercial potatoes became viroid-infected by chance transfer of Mexican papita viroid (MPVd) or related pospiviroid from endemically infected wild solanaceous plants imported as germplasm from Mexico. PSTVd and MPVd share approximately 90% sequence identity, and such plants are known to have been introduced from Mexico to the

United States in the late 19th century in efforts to identify genetic resistance to the potato late blight fungus, *Phytophthora infestans*.

ORGANIZATION AND EXPRESSION OF THE PSTVd GENOME

It is sometimes forgotten that the essential properties of PSTVd were identified without ever seeing its RNA genome as a physical entity. Following sucrose density gradient centrifugation or polyacrylamide gel electrophoresis (the two most powerful analytical techniques available at the time), UV absorbance failed to reveal an RNA species present only in extracts from infected plants. Just 7 years after the discovery of PSTVd and 6 years after its visualization as a discrete UV-absorbing peak in polyacrylamide gel electrophoresis (Diener, 1972), the pioneering phase of viroid research came to an end with the determination of the complete nucleotide sequence of PSTVd-Intermediate (Gross *et al.*, 1978). In the meantime, electron microscopy had shown PSTVd to be a covalently closed circular molecule—the first circular RNA to be described (McClements and Kaesberg, 1977; Sanger *et al.*, 1976)—and evidence for the non-protein-coding nature of viroid genomes had been obtained (Davies *et al.*, 1974; Hall *et al.*, 1974). PSTVd was the first eukaryotic pathogen to have its genome completely sequenced.

Structural studies set the stage for functional genomics

By 1985, the complete sequences of eight viroid species and more than 30 sequence variants had been published. Based on this information, Keese and Symons (1985) proposed that PSTVd and related viroids contain five structural and functional domains. These include (1) a central domain that contains a central conserved region (CCR) capable of forming alternative structures that regulate the replication cycle, (2) a pathogenicity domain, (3) a domain with high sequence variability, and (4 and 5) two terminal domains that are interchangeable between viroid species. Figure 2 shows the location of these five domains as well as several other important structural features of PSTVd. Viroid evolution appears to have involved repeated rearrangement of domains, and the concepts proposed in the study by Keese and Symons have had a profound impact on viroid molecular biology.

Like other single-stranded RNAs, PSTVd can assume a variety of secondary structures. Thanks to studies carried out by Detlev Riesner, Gerhard Steger, and their colleagues, how PSTVd switches between these various structures is well understood—at least *in vitro*. Physicochemical studies of PSTVd started as soon as purified RNA became available, and many aspects of structure formation were under study even before its nucleotide sequence had been determined (for reviews, see Riesner, 1987; Steger and Riesner, 2003). Features of particular interest include alternative

interactions involved in replication-related events [i.e. secondary hairpin II (Loss *et al.*, 1991) and a tetraloop-containing processing structure required for cleavage/ligation of nascent PSTVd plus-strand RNAs (Baumstark *et al.*, 1997)] and a UV-sensitive loop E motif that is also located in the central conserved region (Branch *et al.*, 1985). Very early on, RNA fingerprinting studies revealed that only minor sequence differences distinguish mild from severe strains of PSTVd (Dickson *et al.*, 1979). Many of these differences are located in the pathogenicity domain on the left side of the rod-like secondary structure.

Cress *et al.* (1983) reported the construction of the first infectious viroid cDNA. Recognizing the functional similarities between a longer-than-unit-length PSTVd cDNA and the multimeric RNAs produced during normal RNA-directed rolling circle replication (see below), these investigators inoculated tomato seedlings with a cDNA dimer whose termini were derived from the *Bam*HI site in the upper portion of the central conserved region. Other investigators (e.g. Feldstein *et al.*, 1998; Tabler and Sanger, 1985) later showed that RNA transcripts synthesized from such cDNAs were also infectious. RNA transcripts derived from single sequence variants have been used to study evolution of the PSTVd quasi-species (e.g. Gora-Sochacka *et al.*, 1997; Owens *et al.*, 2002), and site-directed mutagenesis continues to play a key role in studies of PSTVd–host interaction (Zhong *et al.*, 2006).

Replication in the nucleus via an asymmetric rolling circle mechanism

The review by Sanger (1987) provides an excellent overview of early attempts to determine the mechanism of viroid replication. Studies involving ¹²⁵I-labelled PSTVd or citrus exocortis viroid (CEVd) RNA probes (e.g. Grill and Semancik, 1978; Hadidi *et al.*, 1976) began even before the nucleotide sequences of these two viroids had been determined, and there was a great deal of initial confusion concerning the RNA- or DNA-directed nature of the replication process. Early on, bioassays of subcellular fractions derived from PSTVd-infected leaf tissue revealed that PSTVd was concentrated in the nuclear fraction. Introduction of recombinant DNA technology eliminated a number of technical problems related to the purity and/or specificity of the probes used for Northern analysis of replicative intermediates, and by 1984 it was clear that PSTVd and related pospiviroids replicate via an asymmetric rolling circle mechanism in which the only circular species is the incoming infectious plus-strand (Branch and Robertson, 1984).

Synthesis of both plus- and minus-strand PSTVd RNA in highly purified nuclei is inhibited by low levels of α -amanitin, indicating the involvement of host DNA-dependent RNA polymerase II (Muhlbach and Sanger, 1979; Schindler and Muhlbach, 1992). Direct evidence for an association between RNA polymerase II

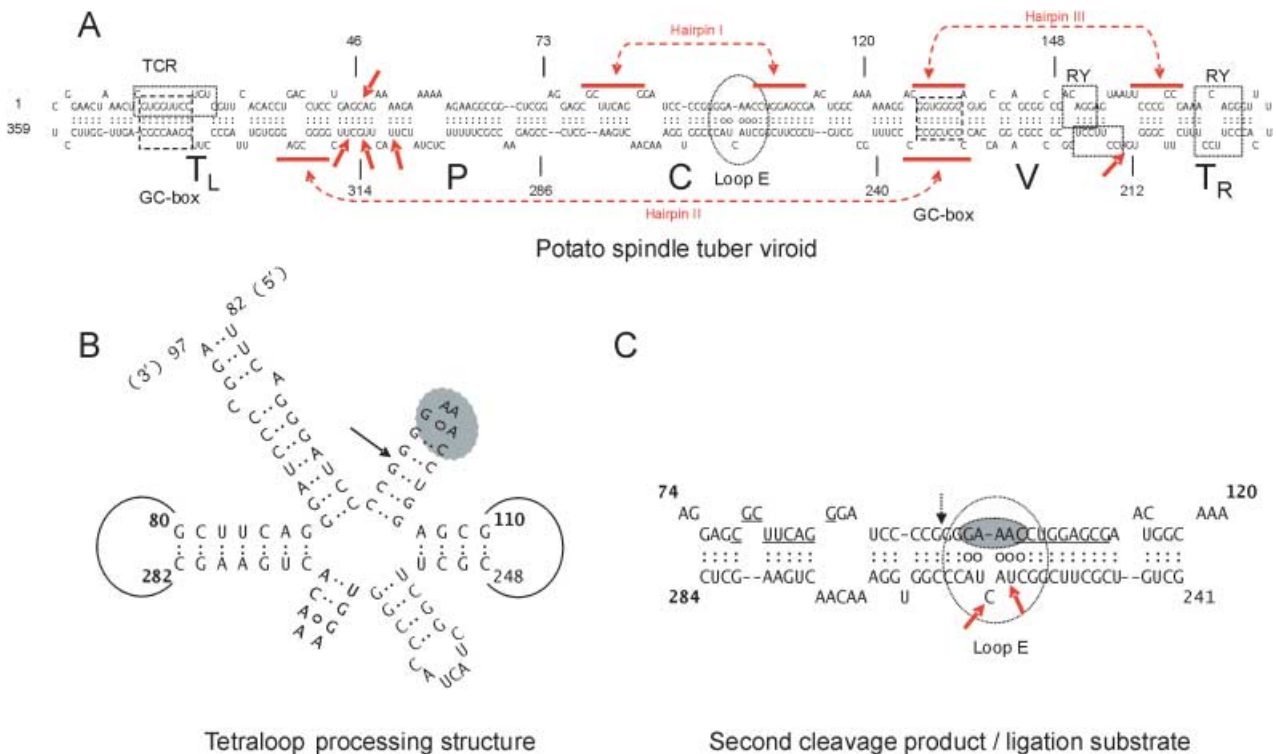


Fig. 2 Structural features of PSTVd. (A) The lowest free energy structure of PSTVd-Intermediate strain showing the boundaries of its five structural/functional domains, i.e. the left (T_L) and right (T_R) terminal loops, the pathogenicity (P) domain, central (C) domain, and variable (V) domain. As indicated by the dashed arrows, disruption of this highly base-paired structure allows three pairs of inverted repeats to interact to form secondary hairpins I–III. Other structural features include (1) two ‘GC-boxes’ (dashed boxes) that may form part of the promoter recognized by RNA polymerase II (Kolonko *et al.*, 2006), (2) two ‘RY motifs’ (dotted boxes) located in the right terminal loop and involved in the interaction with tomato protein VirP1 (Maniatakis *et al.*, 2003), (3) a ‘terminal conserved region’ (TCR) located in the left terminal loop that is of unknown function but highly conserved among all members of the family *Pospiviroidae* except *Hop stunt viroid*, and (4) a bipartite motif that mediates PSTVd transport across tissue boundaries and is defined by a series of sequence changes (arrows) in the P and V domains (Qi *et al.*, 2004). (B) The branched PSTVd processing structure formed by sequences from the central domain prior to the initial cleavage of nascent plus-strand progeny. Note the presence of two GNRA tetraloops. Position of the initial cleavage site is indicated by a small arrow. (C) Structure of the fully cleaved PSTVd monomeric RNA just prior to ligation. Position of the ligation site is indicated by a small arrow. Note the presence of a loop E motif (dashed circle) and a pair of inverted repeats also involved in formation of secondary hairpin I (underlined). Specific sequence changes within loop E (red arrows) dramatically alter the ability of PSTVd to move systemically.

and CEVd was presented by Warrilow and Symons (1999), who showed that addition of a monoclonal antibody directed against the C-terminal domain of the largest subunit of RNA pol II results in immunoprecipitation of a nucleoprotein complex containing both plus- and minus-strand CEVd RNAs. The interaction of PSTVd with other nuclear proteins has been studied using a combination of *in vitro* reconstitution as well as characterization of complexes isolated *in vivo* under non-dissociating conditions (Wolff *et al.*, 1985). Exposure to 0.4 M NaCl disrupted complexes containing unidentified 41-kDa or 31-kDa nuclear proteins but not complexes with histones.

Provided that monomeric progeny can be efficiently excised from the resulting oligomeric intermediates, the rolling circle mechanism eliminates the need for PSTVd replication to initiate at a defined site on the incoming circular template (Diener, 1989). In the case of PSTVd, studies from the Riesner laboratory have

shown that incubation of a longer-than-unit-length PSTVd RNA transcript with a nuclear extract results in specific cleavage between residues G95 and G96 in the upper strand of the central conserved region. The structure of the initial tetraloop-containing processing substrate is shown in Fig. 2(B), and the switch from cleavage to ligation is driven by a change from this tetraloop to a loop E conformation (Baumstark *et al.*, 1997; see Fig. 2C). A long-standing controversy about the initiation site of PSTVd replication appears to have been recently resolved by Kolonko *et al.* (2006), who presented evidence for the initiation of minus-strand synthesis at either position U359 or C1 in the left terminal loop. Hairpin loops provide initiation sites for many of the viral or cellular polymerases that catalyse RNA-directed RNA replication, and more than 20 years earlier Goodman *et al.* (1984) had shown that purified wheat germ RNA polymerase II binds to both PSTVd terminal loops *in vitro*. Additional promoter elements may

include one or both of the 'GC boxes' found elsewhere in the molecule (see Fig. 2A).

Further insight into the mechanism of PSTVd replication has come from *in situ* hybridization studies in which strand-specific probes and confocal microscopy were used to compare the relative distribution of plus- and minus-strand RNAs between the nucleoplasm and the nucleolus. In the first such study (Harders *et al.*, 1989), the highest concentrations of both plus- and minus-strand PSTVd RNAs were found in the nucleoli. Localization of both replicative intermediates and the mature progeny in the nucleolus would appear inconsistent with the proposed involvement of RNA polymerase II in PSTVd replication, but it is possible that either the polymerase or the viroid RNAs could migrate between the nucleoplasm and the nucleolus. A more recent study by Qi and Ding (2003b) indicated that PSTVd minus-strands are localized in the nucleoplasm whereas the plus-strands are distributed between the nucleoplasm and the nucleolus. These data suggest a model in which transcription takes place in the nucleoplasm and oligomeric plus-strand RNAs are transported into the nucleolus for subsequent cleavage and ligation. Identities of the host factors involved in these processes remain to be determined.

For many years, studies of PSTVd replication at the single cell level were hindered by the lack of a reliable protoplast system. Fortunately, this limitation no longer exists thanks to the efforts of Qi and Ding (2002) who described an electroporation protocol that can be used to inoculate protoplasts prepared from tobacco or *Nicotiana benthamiana* suspension culture cells with viroid RNA transcripts. Earlier studies (i.e. Wassenegger *et al.*, 1996) had shown that certain sequence changes are necessary within the loop E motif of the PSTVd-Intermediate strain before the viroid can move systemically in tobacco, and these changes are known to alter the viroid's ability to cross certain tissue boundaries (Zhu *et al.*, 2002). By comparing replication rates in tobacco (restrictive) and *N. benthamiana* (permissive) protoplasts, some (but not all) sequence changes at positions 257 and 259 in the loop E motif were seen to enhance PSTVd replication by five- to ten-fold. Mutational analysis of PSTVd at this level of detail was previously impossible.

Accumulation of progeny is accompanied by RNA silencing

An ever-increasing body of genetic and molecular evidence indicates that higher plants contain at least three distinct pathways for RNA silencing, pathways that provide at least a partial defence against virus infection (Baulcombe, 2004). The existence of a pathway involving DNA methylation and suppression of transcription was first recognized by Wassenegger *et al.* (1994) who were studying PSTVd replication in transgenic tobacco. *De novo* methylation of PSTVd transgene in these plants was dependent on autonomous RNA–RNA viroid replication, thereby suggesting the existence of a mechanism by which host

genes could be targeted and specifically silenced by their own transcripts.

Like many plant viruses and viral satellites, PSTVd replication is accompanied by the appearance of small (21–24 nt) viroid-related RNAs (Denti *et al.*, 2004; Itaya *et al.*, 2001; Papaefthimiou *et al.*, 2001). Late in infection, tomato plants infected with PSTVd exhibit a 'recovery' phenomenon during which PSTVd s(ilencing)i(nduced)RNA concentrations increase prior to a two- to three-fold drop in progeny concentration in the upper leaves (Sano and Matsuura, 2004).

A recent study from the Ding laboratory (Itaya *et al.*, 2007) has shown that (1) PSTVd siRNAs are derived predominantly from the viroid plus-strand, (2) the pathogenicity domain as well as certain other portions of the genome are significantly under-represented in the resulting population of siRNAs, and (3) RNA silencing has little effect on PSTVd replication/accumulation in protoplasts. No evidence was found that PSTVd itself acts as a non-protein suppressor of RNA silencing, and incubation of PSTVd RNA transcripts with a partially purified DICER preparation from *Arabidopsis* resulted in formation of small RNAs containing ~21 nt. Together with the results of two earlier studies (i.e. Matoušek *et al.*, 2004; Wang *et al.*, 2004), these data suggest that the highly base-paired structure of viroids may have evolved to protect them from RISC-mediated degradation.

Consistent with a possible role for PSTVd siRNAs in disease induction, Matoušek *et al.* (2007) have reported that tomato and *N. benthamiana* plants infected with the severe AS1 strain of PSTVd contain higher concentrations of siRNAs than those infected with the mild QFA strain. An earlier study (Itaya *et al.*, 2001) failed to find such a correlation, and evidence for a cause-and-effect relationship is not yet available. We will return to the subject of RNA silencing in a later section outlining possible mechanisms for symptom induction by PSTVd.

Failure to infect Arabidopsis systemically

Arabidopsis offers many potential advantages for studies of viroid–pathogen interactions, but all attempts to transmit PSTVd and other viroids by mechanical inoculation have been unsuccessful. To determine why this might be so, Daròs and Flores (2004) transformed *Arabidopsis* with cDNA constructs expressing dimeric plus-strand transcripts of representative members of the families *Pospiviroidae* and *Avsunviroidae*. Correct processing to the circular monomer was always observed, thereby demonstrating the presence of the necessary RNase and RNA ligase activities. Northern blot hybridization also revealed the presence of multimeric minus-strand CEVd and HSVd RNAs, showing that the first RNA–RNA transcription of the rolling-circle mechanism occurs in *Arabidopsis* and that the resulting multimeric minus-strand RNAs remain unprocessed. Because plants expressing a dimeric minus-strand HSVd transcript also contained low levels of monomeric circular plus-strand RNA, *Arabidopsis* is also able to

support the second phase of RNA–RNA transcription. And finally, *Agrobacterium*-mediated inoculation of *Arabidopsis* with three different dimeric pospiviroid cDNAs revealed that none of these viroids could move out into distal parts of the plant. It appears that infectivity in *Arabidopsis* is limited by some as-yet-unidentified deficiency in PSTVd movement and/or replication.

The fact that only single C/U substitution in the loop E motif of the PSTVd-Intermediate strain is sufficient to allow free movement in tobacco (Qi and Ding, 2002; Wassenegger *et al.*, 1996) suggests that certain rare variants in the PSTVd quasispecies could, perhaps, systemically infect *Arabidopsis*. A recent study by Matoušek *et al.* (2004) has shown that thermal stress of PSTVd-infected *N. benthamiana* leads to appearance of a broad distribution of sequence variants where most of the mutations accumulate in the pathogenicity domain and other portions of the left side of the molecule. This pool of viroid 'thermomutants' was transcribed into cDNA and used for biolistic inoculation of *Raphanus sativa*, where PSTVd infection was detectable by RT-PCR. Newly generated inoculum from *R. sativa* was again transcribed into cDNA and transferred to *Arabidopsis* by biolistic inoculation. Progeny levels in *Arabidopsis* infected with populations of PSTVd variants were approximately 300 times lower than in tomato, but the progeny were able to move away from the inoculation site. Sequencing studies revealed a mixture of major and minor variants different from that of the original thermomutant population isolated from *N. benthamiana*. Although these studies are promising, and stress-mediated shifts in quasispecies composition could play a significant role in viroid adaptation to new hosts, no PSTVd variants that are highly infectious for *Arabidopsis* have yet been identified.

Transport of PSTVd in infected plants

The vascular system of higher plants provides a long-distance network that communicates environmental inputs sensed by mature organs to meristematic regions of the plant (Lough and Lucas, 2006). In addition to photoassimilates and other small molecules, the phloem translocation stream contains a variety of macromolecules, including mRNA, small RNA and proteins. Pioneering studies on plant viruses revealed the existence of viral-encoded movement proteins that are able to move from cell-to-cell via the plasmodesmata. These movement proteins bind RNA/DNA in a sequence non-specific manner to form nucleoprotein complexes that are delivered to the plasmodesmata with the assistance of host proteins and the cytoskeleton. Viruses have evolved a combination of movement and ancillary proteins that work in concert to create stable nucleoprotein complexes that can compete with endogenous complexes for the plasmodesmatal trafficking machinery (Lucas, 2006).

Like most viruses, PSTVd moves from source to sink in the phloem of infected plants (Palukaitis, 1987); in order to establish

a systemic infection, however, PSTVd must navigate these pathways without benefit of viroid-encoded movement protein(s). Two recent reviews by Biao Ding and colleagues (Ding *et al.*, 2005; Ding and Itaya, 2007) have described in detail how various aspects of viroid movement are being used as model systems in which to study much broader questions in RNA biology. Here, we focus on the increasing evidence that known tissue tropisms and host range restrictions affecting PSTVd may involve incompatibilities between specific sequence motifs in the viroid and host proteins involved in RNA trafficking.

The story begins with one of the first mutants created by site-directed mutagenesis of PSTVd cDNA, a mutant containing several changes in the right terminal domain (Hammond and Owens, 1987). Although non-infectious when mechanically inoculated on to the cotyledons of young tomato seedlings, this mutant was infectious when introduced into the stem by *Agrobacterium*-mediated inoculation (Hammond, 1994). PSTVd replicative intermediates and progeny were restricted mainly to gall and root tissues, and progeny were only occasionally detectable in the newly developing leaves. Rather than abolishing replication *per se*, mutations in the right terminal domain of PSTVd appear to alter interaction with specific host components, thereby disrupting the normal pattern of intercellular transport of the viroid or limiting its replication to specific cell types. Studies by Maniataki *et al.* (2003) later showed that these mutations interfere with the ability of PSTVd to interact with a bromodomain-containing protein from tomato known as VIRP1. Locations of the two binding sites for VIRP1 are shown in Fig. 2(A).

The first direct evidence that PSTVd contains one or more RNA transport motifs came from microinjection studies carried out by Ding *et al.* (1997) who showed that fluorescently labelled PSTVd RNA is able to move freely from cell to cell in the mesophyll of tobacco where it accumulates in the nuclei. Significantly, PSTVd was also shown to function in *cis*, conferring upon an otherwise non-mobile RNA the ability to move from cell to cell. Using a permeabilized protoplast system, Woo *et al.* (1999) later showed that PSTVd import into the nucleus is a specific and regulated process that does not require an intact cytoskeleton. To identify the sequence motif(s) involved in nuclear import, Zhao *et al.* (2001) developed a potato virus X-based, whole plant assay that uses green fluorescent protein (GFP) as the reporter molecule. The intron-containing subgenomic GFP mRNA that is synthesized by a specially modified PVX vector cannot produce a functional GFP unless it is imported into the nucleus, where the intron can be removed and the spliced RNA returned to the cytoplasm. The appearance of green fluorescence in leaf tissues inoculated with PVX-GFP constructs containing a full-length PSTVd cDNA embedded in the intron indicated that nuclear import and RNA splicing events did occur. By replacing the full-length PSTVd cDNA with various smaller portions of the PSTVd genome, certain sequence/structural elements within the upper strand of the central conserved

region have subsequently been identified as important determinants of nuclear transport (R. Hammond, personal communication).

A series of *in situ* hybridization studies carried out by Ding and colleagues have examined the ability of PSTVd to move from cell to cell and across tissue boundaries. Among the early conclusions of these studies: First, the phloem is able to recognize and control PSTVd entry into specific sink organs (Zhu *et al.*, 2001) and, second, the phloem entry and exit processes appear to be differentially regulated (Zhu *et al.*, 2002). The effects of two mutations in the loop E motif were examined in some detail, and both were found to block PSTVd movement from the phloem to the surrounding non-vascular tissue. Further insight into the nature of this differential regulation was obtained by Qi *et al.* (2004) who showed that PSTVd movement from bundle sheath to mesophyll is controlled by a bi-partite sequence motif. Interestingly, this motif is not required for transport in the opposite direction, and regulation is observed only in young leaves. The five sequence changes that define this motif are distributed between the pathogenicity (four changes) and variable (one change) domains (see Fig. 2A), but its nature as well as the identity of the host proteins presumably involved in the recognition process remain to be determined.

The ease with which phloem exudate can be obtained from cucurbit species such as cucumber (*Cucumis sativus* L.) has made HSVd the viroid of choice for biochemical studies of long-distance viroid transport. Phloem lectin PP2, the most abundant protein in phloem exudate, has been shown to interact with HSVd *in vitro* (Gómez and Pallas, 2001; Owens *et al.*, 2001), and further characterization of CsPP2 revealed that this protein (1) contains a binding motif for double-stranded and/or highly structured RNA and (2) can traffic from rootstock to scion in cucumber-pumpkin heterografts (Gómez and Pallas, 2004). Although the data available are highly suggestive of an important role for PP2 in long-distance viroid movement, direct evidence for the involvement of PP2 (and possibly other phloem proteins) is currently lacking.

DISEASE INDUCTION

In the absence of viroid-encoded polypeptides, the dramatic symptoms often associated with PSTVd infection must result from the direct interaction of host cell components with one or more viroid-related RNAs. While early studies focused on the possible involvement of plus-strand PSTVd genomic RNA in symptom induction, the results of more recent studies point to a key role for RNA silencing. Several lines of evidence now indicate that pathogenicity is a complex process involving sequences distributed throughout the PSTVd genome—and not just those located in the so-called pathogenicity domain.

Studies of PSTVd pathogenicity have been routinely carried out in the tomato cultivar 'Rutgers' or other sensitive varieties that respond strongly to PSTVd infection under greenhouse conditions.

Earlier field studies carried out in potato had identified a number of PSTVd strains causing very severe tuber symptoms (e.g. the 'unmottled curly dwarf' strain; Diener, 1987), but many of these strains were lost before modern sequencing methods became widely available. Thus, the terms 'mild', 'intermediate' and 'severe/lethal' do not necessarily reflect the symptoms seen in the natural host under field conditions.

Even before the complete nucleotide sequence of the PSTVd-Intermediate strain had been determined, RNA fingerprinting studies revealed the sequences of mild and severe strains to be almost identical (Dickson *et al.*, 1979). To explain how as few as 1–2 substitutions located in one small portion of the molecule could result in such dramatic changes in its biological properties, Schnölzer *et al.* (1985) proposed the existence of a 'virulence modulating region' whose stability would influence the interaction with unidentified host cell components. Although an attractive hypothesis that incorporated a great deal of biophysical information, this proposal did not explain the variation in symptom expression observed for a second pospiviroid (i.e. CEVd; Visvader and Symons, 1985). Furthermore, quantitative comparisons of symptom development and progeny accumulation for a series of novel pospiviroid chimeras revealed the existence of at least three discrete pathogenicity determinants (Sano *et al.*, 1992). Comparison of the two parental viroids (i.e. CEVd and tomato apical stunt viroid) revealed regions of sequence and/or structural variability in the left terminal, pathogenicity, and variable/right terminal domains corresponding to these genetic determinants.

Recently, the loop E motif located in the central domain of PSTVd has also been shown to play an important role in regulating symptom production. Effects of single nucleotide substitutions at positions 257 or 259 on host range (specifically, on the viroid's ability to enter/exit the vascular system) were considered in the previous section on transport. Interestingly, the presence of one of these changes (i.e. a U/A substitution at position 257) also leads to a striking 'flat top' phenotype in tomato (Qi and Ding, 2003a). Neither PSTVd replication levels nor tissue tropism were affected by the change at position 257, and this new pathogenicity determinant appears to function independently of the nearby 'classic pathogenicity domain'.

It is not yet clear how the nucleotides which comprise these PSTVd pathogenicity determinants interact with as-yet-unidentified host components to induce disease. Loop E motifs in ribosomal RNAs often act as recognition sites for RNA–protein interaction (Allison *et al.*, 1991), and the same may well be true for the loop E motif of PSTVd. Many plants contain so-called ribosome-inactivating-proteins (RIPs), and the binding sites for several well-characterized RIPs include a loop E motif. Sharma *et al.* (2004) have described the isolation of a tobacco RIP that also has superoxide dismutase activity. Could the ability of this protein to interact with the loop E motif of PSTVd explain the effect of certain sequence changes on host range and symptom expression?

As described by Zhong *et al.* (2006), there is a wealth of information about the structure of loop E including detailed isostericity matrices for non-Watson–Crick base pairs that can be used to guide future mutational and biochemical studies, and direct evidence for the existence of loop E motif *in vivo* has recently been published (Eiras *et al.*, 2007; Wang *et al.*, 2007). Secondary/tertiary structure may also control the interaction of host proteins with the nearby pathogenicity domain (Owens *et al.*, 1996; Schmitz and Riesner, 1998). Here, potential candidate proteins include protein kinase(s) similar to the mammalian interferon-induced, dsRNA-specific protein kinase shown to be differentially activated by PSTVd strains of varying pathogenicity (Diener *et al.*, 1993).

A likely role for RNA silencing in viroid pathogenicity

Recently, this focus on direct interaction between the PSTVd genome and host cell components has begun to shift. The presence of small PSTVd-related siRNAs in infected plants has led several groups to speculate about a possible role for RNA silencing in symptom induction. The strongest evidence for the involvement of RNA silencing comes from a study by Wang *et al.* (2004) who reported that transgenic tomatoes expressing a non-replicating hairpin RNA derived from PSTVd exhibit stunting and epinasty similar to that observed in infected plants. BLAST searches of the Arabidopsis (<http://www.arabidopsis.org>) and Tomato Genome Databases (<http://www.sgn.cornell.edu>) reveal numerous potential targets for PSTVd-directed RNA silencing (Shao and Owens, unpublished data), many of which involve an oligo(A) sequence located in the upper portion of the pathogenicity domain (see Fig. 2A). Several recent reports indicate that the situation may be more complex than initially thought, however. For example, a recent analysis of siRNAs recovered from PSTVd-infected tomato indicates that sequences derived from the pathogenicity domain are severely under-represented (Itaya *et al.*, 2007); also, efforts to compare the relative concentration of viroid-related siRNA in plants infected with mild or severe strains of PSTVd have yielded contradictory results (Itaya *et al.*, 2001; Matoušek *et al.*, 2007).

As yet, no direct evidence has been presented for PSTVd-mediated cleavage/inactivation of specific host mRNAs. Several years ago, macroarray analysis involving 1156 cDNA clones recovered from a subtracted tomato cDNA library identified a total of 55 genes whose regulation is specifically altered by PSTVd infection (Itaya *et al.*, 2002). The corresponding gene products are involved in a variety of cellular functions, e.g. defence/stress responses, cell-wall structure, chloroplast function and protein metabolism. Transcript levels for most of these genes were up-regulated by PSTVd infection, and only a small minority (< 15%) were down-regulated. In unpublished experiments, we have obtained similar results using the much larger Tom2 cDNA microarray available from the Center for Gene Expression

Profiling at the Boyce Thompson Institute (<http://bti.cornell.edu/CGEP/CGEP.html>). In this case, approximately 10% of all genes appeared to be up-regulated 3–4 weeks post inoculation, while only 66 genes (< 1%) were down-regulated. The limitations of microarray analysis are well known (Larkin *et al.*, 2006; Whitham *et al.*, 2006), and currently available tomato arrays do not include several genes such as a 55-kDa protein kinase (Hammond and Zhao, 2000) and RNA-dependent RNA polymerase (Schiebel *et al.*, 1998) known to be induced by PSTVd infection. Nevertheless, expression profiling methods are likely to play a key role in future attempts to map host responses to PSTVd infection.

Interaction of PSTVd with host proteins

With the exception of tomato protein VIRP1 whose binding site in the right terminal domain has been well characterized (Gozmanova *et al.*, 2003), very little is known about PSTVd interaction with host proteins. In some cases (e.g. the presumed involvement of the phloem lectin PP2 in long-distance phloem transport), interaction appears to be surprisingly non-specific; other interactions (e.g. Dicer-mediated cleavage of PSTVd) may be more specific. Viroid infection leads to the accumulation of several pathogenesis-related proteins (Gadea *et al.*, 1996; Itaya *et al.*, 2002), and evidence has been presented that PSTVd infection stimulates the phosphorylation of a 68–70-kDa tomato protein associated with double-stranded RNA-stimulated protein kinase activity (Hiddinga *et al.*, 1988; Langland *et al.*, 1995). In support of a potential role for this enzyme in an infection-related signalling cascade, incubation of its mammalian homologue (i.e. pKR) with PSTVd strains of varying pathogenicity leads to differential activation (Diener *et al.*, 1993). Unfortunately, however, all efforts to clone the gene encoding this kinase have been unsuccessful. More recently, Hammond and Zhao (2000) have characterized a second protein kinase whose expression is up-regulated by PSTVd infection. The role of these and other protein kinases in modulating PSTVd–host interaction remains to be determined.

FUTURE DIRECTIONS

The pervasive influence of computers and other electronic devices on modern life has given rise to a ‘Simplicity Paradox’—the desire for devices that are simple and easy to use, yet able to do all the complex things we might ever want. Indeed, a book entitled *The Laws of Simplicity* by MIT professor John Maeda offers ten laws for balancing simplicity and complexity. If, for a moment, we consider pathogens to be biological devices, it becomes clear that Diener’s (1971) formulation of the viroid concept offers a solution to this paradox. The wealth of molecular information accumulated over the last 35 years may tend to obscure the underlying simplicity, but the outlines of a simpler, more elegant explanation for the ability of PSTVd (and other viroids) to replicate autonomously and cause disease has begun to emerge.

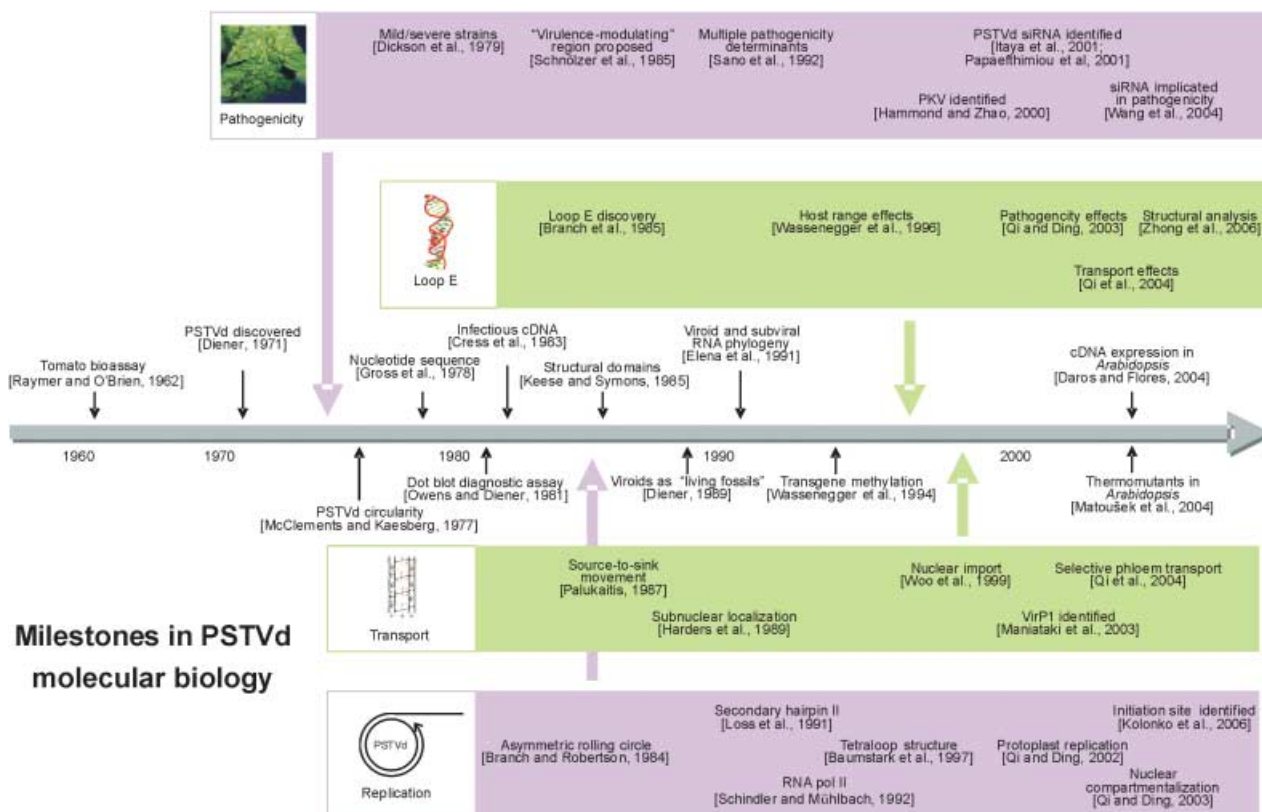


Fig. 3 Milestones in PSTVd molecular biology. Key findings in three areas (pathogenicity, replication, and transport) are grouped in shaded boxes to highlight changes in experimental approach/interpretation. The best-characterized structural element in PSTVd is the loop E motif that plays an important role in all three aspects of viroid–host interaction. Key conceptual and technical advances are arranged along the central timeline.

To understand how this is so, it is helpful to arrange the key studies involving PSTVd along a timeline. As shown in Fig. 3, this timeline begins with the development of the tomato bioassay by Raymer and O'Brien (1962) and ends (temporarily) with the mutational analysis of loop E structure by Zhong *et al.* (2006). Key conceptual and technical advances such as Diener's discovery of PSTVd (1971), identification of its five structural/functional domains (1985) and demonstration of PSTVd cDNA infectivity (1983) are shown nearest the timeline. Three of the horizontal groupings arranged above and below the timeline contain studies related to specific aspects of viroid/host interaction; namely, replication, transport and pathogenicity. The fourth grouping contains studies involving a single structural element, the loop E motif of PSTVd.

In contrast to studies of PSTVd replication and pathogenicity which began as the pioneering era in viroid research was drawing to a close, detailed studies of PSTVd movement did not begin until the mid 1990s. These studies of the intra- and intercellular movement of PSTVd marked a significant shift in emphasis from the molecular genetics of replication/pathogenicity *per se* to the cell biology of viroid–host interaction. This shift began with

studies of PSTVd transport into the nucleus and across various phloem-associated tissue boundaries and has expanded to include the role of RNA silencing and other defence/signalling systems in modulating viroid–host interaction. Individual studies have become broader in scope and often combine several experimental approaches.

A recent series of studies from the Ding laboratory have shown that the PSTVd loop E motif plays an important role in many different aspects of viroid–host interaction. In particular, Zhong *et al.* (2006) have shown how detailed structural information obtained from non-Watson–Crick base pairings in the loop E motifs of 5S rRNA and other non-viroid RNAs can be used to guide mutational analysis of loop E function in PSTVd replication. In this study, the effects of carefully chosen mutations were evaluated using a combination of *in vivo* and *in vitro* assays. The loop E motif is a particularly challenging place to begin structure/function analysis because of the number and complexity of the structural rearrangements that occur during replication. Fortunately, biophysical studies from the Riesner laboratory provide detailed insight into the dynamics of the cleavage/ligation process. And these challenges do not end with the loop E motif.

As shown in Fig. 2, certain of the sequences involved in the breakdown of the entire native structure via formation of secondary hairpins I–III are located within one of two 'GC boxes' that may be part of the promoter recognized by RNA polymerase II. Determining how and in what order these overlapping structural elements form and break down, thereby allowing PSTVd to complete its replication cycle, move from cell to cell and, in some cases, cause disease will present even greater challenges.

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